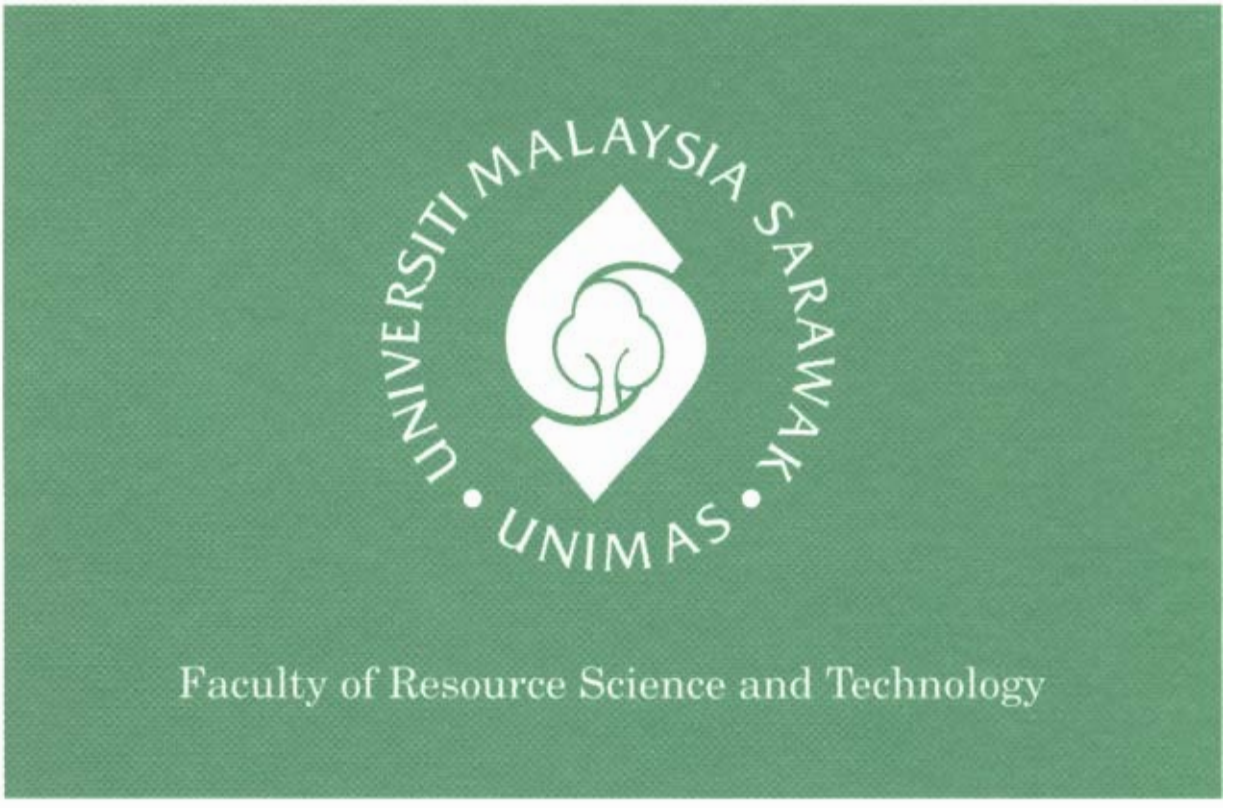

A STUDY ON THE PHYLOGENETIC RELATIONSHIP OF *TUPAIA TANA* AND *T. MONTANA* INFERRED FROM MITOCHONDRIAL CYTOCHROME OXIDASE I (COI) GENE

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This project is submitted in partial fulfillment of the requirements for the degree of Bachelor of Science with Honors
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A Study on the Phylogenetic Relationship of *Tupaia tana* and *T. montana* Inferred from Mitochondrial Cytochrome Oxidase I (COI) Gene

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Abstract

A phylogenetic relationship study was aimed among the genus *Tupaia* based on the cytochrome oxidase I (COI) gene. Five species were sampled namely *T. montana*, *T. tana*, *T. gracilis*, *T. glis*, and *T. minor*. The samples were collected from the Kubah National Park, Universiti Malaysia Sarawak (UNIMAS) campus and Zoological Museum of UNIMAS. However, only *T. tana* and *T. montana*, and one outgroup, *Callosciurus notatus* were successfully sequenced used for the phylogenetic tree reconstruction. Both Neighbor-Joining (NJ) and Maximum Parsimony (MP) methods produced similar phylogenetic tree topologies consisting of two major clades. Clade 1 consisted of *T. montana* while clade 2 consisted *T. tana*. In general, the analysis of the mtDNA COI gene was useful to resolve interspecific phylogenetic relationship among the selected species of the genus *Tupaia*.

Keywords: cytochrome oxidase I, genus *Tupaia*, mitochondrial DNA, molecular phylogeny, sequencing analysis.

Abstrak:

Kajian hubungkait filogenetik dikalangan genus *Tupaia* telah dibina menggunakan jujukan sitokrom oksida I (COI) pada gen mitokondrial. Sebanyak lima spesis digunakan dalam kajian ini iaitu *T. montana*, *T. tana*, *T. gracilis*, *T. glis*, dan *T. minor*. Semua sampel adalah diperolehi dari Taman Negara Kubah, kampus Universiti Malaysia Sarawak (UNIMAS) dan dari koleksi Muzium Zoologi UNIMAS. Tetapi, hanya T. *tana* dan *T. montana*, dan satu species kumpulan terkecuali, *Callosciurus notatus* sahaja yang telah berjaya dijujukkan untuk menghasilkan dalam pembinaan pokok filogenetik. Kaedah "Neighbor-Joining" dan "Maximum Parsimony" telah menghasilkan dua pokok filogenetik yang serupa dan mengandungi dua kumpulan utama. Kumpulan pertama mengandungi *T. montana* manakala kumpulan dua mengandungi *T. tana*. Secara keseluruhannya, analisis jujukan COI pada mitokondrial DNA didapati berhasil dalam mengungkapkan hubungkait filogenetik antara spesis- spesis yang terpilih dalam genus *Tupaia*.

Kata kunci: sitokrom oksida I, genus *Tupaia*, mitokondrial DNA, filogeni molekular, analisis jujukan.

1.0 Introduction

1.1 Background

Treeshrews resemble small tree squirrels but differ in many details of anatomy and behaviors (Feldhamer *et al.*, 1999). They have a very long muzzle with a total of 38 teeth, all which are pointed rather than chisel-like incisors (Payne *et al.*, 1985). Treeshrews are small bodied, active and alert mammal (Martin *et al.*, 2001). Most species are predominantly arboreal, diurnal and omnivorous, feeding on both fruits and insects (Payne *et al.*, 1985). Their primitive morphology allows them to be proficient at running, jumping, and climbing on many substrates (Mitchell, 2006). Treeshrews do not seem to be closely related to any extant taxon (Mitchell, 2006).

Treeshrews were inhabit in one of the most geographically and geologically complex regions of the world (Audley-Charles, 1987; George, 1987; Whitmore, 1987). They are widely distributed at the west of the Wallace's line and not extending to Sulawesi, Lombok or islands eastward of these (Musser, 1987; Kithener *et al.*, 1990). According to Feldhamer *et al.* (1999), treeshrews are restricted to the Oriental faunal region, ranging from India, Southern China, and the Philippines southward through Borneo and the Indonesian island. The treeshrews come from the order Scandentia which contain a single family, Tupaiidae, five genera and sixteen species (Vaughan, 1986; Corbet and Hill, 1992). The Tupaiidae which is endemic to the Indomalayan region (Corbet and Hill, 1992) are divided into two subfamily Ptilocercinae and Tupaiinae (Olson *et al.*, 2005).

The subfamily Ptilocercinae contains single genus and species namely *Ptilocercus lowii* which is also known as Pen-tailed treeshrew (Corbet and Hill, 1992; Olson *et al.*, 2005). The most basal treeshrew *P. lowii* is the living treeshrew that most closely resembles the ancestral scandentian in both its ecology and its morphological attributes (Olson *et al.*, 2004). Besides that, *P. lowii* is nocturnal insectivorous and foraging mainly in the canopy and on the ground of the forest (Corbet and Hill, 1992), but also has been recorded in gardens (Payne *et al.*, 1985). Another significant different of *P. lowii* from other members of the order is that it has longer dark eye stripe, narrower muzzle, greyer pelage and does not resemble squirrel (Payne *et al.*, 1985). The *P. lowii* is well distributed ranging from Peninsular Malaysia, Thailand, Sumatra and adjacent island (Payne *et al.*, 1985). In Borneo, *P. lowii* is known to be found in the lowlands of Sabah and Sarawak, from Sandakan in the north-east to Kuching in the south-west, as well as Labuan Island (Payne *et al.*, 1985).

The subfamily Tupaiinae consisting of four genera namely *Urogale*, *Anathana*, *Dendrogale* and *Tupaia* (Corbet and Hill, 1992). According to Corbet and Hill (1992), the genera *Urogale* and *Anathana* both contains single species namely *U. everetti* (Mindanao treeshrew) and *A. ellioti* (Madras treeshrew) respectively. Besides that, Corbet and Hill (1992) also listed that the genus *Dendrogale* contains two species namely *D. murina* (Northern smooth-tailed treeshrew) and *D. melanura* (Bornean smooth-tailed treeshrew). Lastly, the authors stated that there are 11 species of *Tupaia* endemic to Indomalayan region and 8 species are found in Borneo.

1.2 Statement of Problems

According to Feldhamer (1999), Tupaiids have had a conflicting taxonomic history which at various time being placed in the order primates and insectivores, and currently within the order Scandentia. The phylogenetic relationships of the order Scandentia has been explored in both morphological and molecular studies but remains a debatable issue (Olson *et al.*, 2004). Their systematic studies are scarce and the taxonomy is poorly resolved. The current systematic classification of the treeshrews only examined at the generic level within the order Scandentia where the relationships among the species within each genus are poorly discussed (Olson *et al.*, 2004).

Olson *et al.* (2004) had reconstructed the phylogenetic relationship among treeshrews based on several published studies of qualitative morphological variation among living treeshrews for assessing intergeneric relationship. However, reanalysis of the data from each of the studies demonstrates that none of the trees constructed in the original publications represent the most parsimonious interpretation and failed to resolve either intergeneric or interspecific relationships (Olson *et al.*, 2004). According to the author, there are also several inconsistencies and conflicts with respects to character coding were occurred. For example, Butler (1980) considered hypocone development as a single character with essentially identical states and codings among genera. On the other hand, Steele (1973) characterized the condition of the hypocone separately with varying combinations of states among different taxa. Furthermore, the molars of *T. minor* and *T. gracilis* are noted to lack hypocones, yet *T. minor* was coded as having poorly developed hypocones and those *T. gracilis* were coded as being well developed (Olson *et al.*, 2004).

The first investigation of treeshrews (order Scandentia) phylogenetic was done by Olson *et al.* (2005) based on DNA sequences, utilizing previously published sequences from the mitochondrial 12S rRNA gene and combining them with newly generated sequence data from 15 species. The first molecular study of treeshrews had revealed the relationship among the genera within the order Scandentia but poorly examined the relationship among the species.

Therefore, realizing the importance of *Tupaia* species as a potential seed disperser and as insect predators besides the need for the conservation, a comprehensive study on their systematics is essential for appropriate management and conservation of small mammal biodiversity (Payne *et al.*, 1985 and Emmons, 1992).

Since the phylogenetic relationship based on morphological data is confusing and have neither resolved the treeshrews' intergeneric nor interspecific relationship, the phylogenetic relationship study based on molecular evidence would be an effective way to classify and identify the genus *Tupaia* (Olson *et al.*, 2004). In fact, the development of molecular techniques has helped invigorate studies in genetics, taxonomy and systematics for resolving problems in the systematics and the classification of taxa (Wallis and Arntzen, 1989).

According to Olson *et al.* (2004), DNA sequence data will undoubtedly provide a better insight into treeshrew interrelationships. Thus, DNA sequencing method was used in this study to detect genetic variation among the species within the genus *Tupaia*. Six species were examined namely *T. montana*, *T. tana*, *T. gracilis*, *T. glis*, *T. minor*, and finally *Callosciurus notatus* as outgroup. Mitochondrial cytochrome oxidase I (COI) gene was amplified by using polymerase chain reaction (PCR).

1.3 Objectives

The main objective of this study was to reconstruct the phylogenetic relationship of the genus *Tupaia* inferred from mtDNA COI gene in order to resolve the taxonomic problem regarding the genus.

2.0 Literature Review

2.1 Genus *Tupaia*

Most of *Tupaia* species resemble long-nosed squirrel and diurnal mammal (Martin *et al.*, 2001). Their facial vibrissae are short and the pinnae are low and rounded (Martin *et al.*, 2001). Their habitats include forested areas and brushy, rocky slopes due to their behavior of liking to shelter in holes between rocks or roots, and in the tree hollows (Martin *et al.*, 2001). They can be found throughout the Indomalayan region (Corbet and Hill, 1992). The species within the genus are classified based on the morphological characteristics by referring to the Payne *et al.* (1985).

All *Tupaia* species are dependent on fruits and invertebrates as their diet (Feldhamer, 1999). Feldhamer (1999) also mentioned that the treeshrew do little damage to crops or plantations. Even though the area of forest habitat continue to become less, none of the species of treeshrews are listed endangered (Feldhamer, 1999).

Corbet and Hill (1992) listed 11 species representing the genus *Tupaia* that are endemic to the Indomalayan region. According to Payne *et al.* (1985), there are only eight species found in Borneo, namely, *T. glis* (Common treeshrew), *T. splendidula* (Red-tailed treeshrew), *T. montana* (Mountain treeshrew), *T. minor* (Lesser treeshrew), *T. gracilis* (Slender treeshrew), *T. picta* (Painted treeshrew), *T. tana* (Terrestrial treeshrew), and *T. dorsalis* (Striped treeshrew).

Payne *et al.* (1985) described that *T. glis* has banded dark and pale hairs on upper parts and usually has a pale stripe on each shoulder. *T. glis* is most often seen active around fallen trees,

in low woody vegetation or on the ground (Payne *et al.*, 1985). Besides that, this species also has been recorded at the height of up to 1100 m in the Kelabit uplands (Payne *et al.*, 1985). According to Payne *et al.* (1985), *T. splendidula* is a plain reddish tree shrew with pale orange stripe on the shoulder if present and has pure dark red hairs on the upperparts of the tail, orange below the tail. *T. splendidula* is similar with *T. glis* which is slightly larger and more uniformly colored above with banded hairs on the tail (Payne *et al.*, 1985).

According to Payne *et al.* (1985), *T. minor* has a thin tail and a reddish tinge towards the rear. It is proposed that arboreal Tupaiids, such as *T. minor*, may represent better models for early primates than didelphid marsupials because they are capable of grasping and are more closely related to primates (Sargis, 2001). *T. minor* is similar to *T. gracilis* which has no reddish tinge and it tend to have a bushy tail, but they can be distinguished by hindfoot or skull measurements (Payne *et al.*, 1985). Payne *et al.* (1985) also had mentioned that *T. picta* is similar to *T. tana* but *T. picta* is smaller with a relatively shorter muzzle.

T. montana usually appears entirely dark brown when glimpsed in the field (Payne *et al.*, 1985). Besides that, Payne *et al.* (1985) also stated that *T. montana* is one of the commonest mammals in primary montane forests in Sabah. According to Payne *et al.* (1985), *T. dorsalis* has a thin, black line running from the neck almost to the base of the tail diagnostic.

2.2 Mitochondrial DNA (mtDNA) cytochrome oxidase I gene

MtDNA COI gene is a subunit from cytochrome oxidase complex that is part of the transport chain (Palumbi, 1996). COI is protein coding region gene (Palumbi, 1996). COI align sequences to one another easily because its amino acid sequences are highly conserved across phyla (Palumbi, 1996; Staton, 2003). Amino acid sequences are useful in phylogenetic reconstruction of evolutionary branches (Palumbi, 1996). COI is useful in amplification of every phylum attempted except Cnidaria (Palumbi, 1996). According to Dasmahapatra and Mallet (2006), COI gene have been successfully identified individuals down to species level with a success rate ranging from 98% to 100% in North American birds (Hebert *et al.*, 2004b) and most recently in tropical Lepidoptera (Hajibabaei *et al.*, 2006). Besides correctly identifying known species, a number of probably cryptic species also have been discovered (Dasmahapatra and Mallet, 2006).

3.0 Materials and Methods

3.1 Sample Collection

The samples were collected from fieldwork conducted in Kubah National Park which had started from 26 August 2006 until 2 September 2006. Samples were also collected from UNIMAS secondary forest near the Alamanda College. Additional samples were also taken from the UNIMAS Zoological Museum where the samples were collected from all over Sarawak.

There were about 70 to 80 cage traps used to capture the treeshrews. Treeshrews were identified based on Payne *et al.* (1985). Voucher specimens were killed using chloroform and the muscle tissues were excised and immersed in 90% ethanol inside 1.5 ml cryovials. All the equipment used to handle the sample was steriled. The specimen was kept in -20°C for long-tem storage.

3.2 Laboratory Methods

3.2.1 Extraction of genome DNA

Total DNA was extracted from muscle tissues using standard cetyl-tri-methyl ammonium bromide (C-TAB) protocol following Grewe *et al.* (1993). Approximately 1 cm tissue samples were minced using the sterilized dissect kits and transferred into the 1.5 ml microcentrifuge tube containing 700 µl of 2X C-TAB. This was then added with 6 µl of Proteinase-K (100 mg/ml). The mixtures were incubated in water bath at 60°C until the tissues were fully digested. After incubation, 700 µl of chloroform isoamyl-alcohol was added and was mixed

by inverting the tube several times and subsequently centrifuged at 13 000 revolutions per minute (rpm) for 15 minutes. Approximately 600 µl of the upper aqueous layer or supernatant were collected using Gilson micropipette with disposable tips and transferred into another sterile 1.5 ml microcentrifuge tube. After that, 600 µl of absolute ethanol was added to the transferred supernatant and was vortexed to mix the reaction thoroughly. The tube was centrifuged again at 13 000 rpm for 15 minutes. The nucleic acid was pelleted by the centrifugal force inside the inner surface of the tube. The remaining supernatant was discarded carefully. The pellet were washed and precipitated with 600 µl of 70% ethanol and 25 µl of sodium chloride (3 M NaCl). The tube was vortexed again and centrifuged at 13 000 rpm for 15 minutes. The ethanol was discarded and the pellet was air-dried. Finally, about 25 µl of distilled water (dH_2O) were added to the remaining pellet in the tube.

Two microlitres (2 µl) of the DNA product was visualized by gel electrophoresis of 1% agarose gel containing 0.8 µl of ethidium bromide (EtBr). One microlitre (1 µl) of 6X load dye (Promega or Fermentas) was mixed with the 2 µl of genomic DNA and 1 kb DNA ladder (GeneRuler™, Fermentas). The electrophoresis was performed for 40 to 45 minutes at 94 V and visualized under UV transluminator and photograph was printed out from the Biorad Documentation System.

3.2.2 Polymerase Chain Reaction

The mtDNA COI gene was amplified by PCR in an Eppendorf Mastercycler machine. The combination of primers to amplify 500 bp of COI is shown in Table 1 (Palumbi, 1996). Table 2 shows the PCR ingredients of a 25 µl reaction while Table 3 shows the PCR cycle parameter.

Table 1: The sequences for the primer cytochrome oxidase 1 (Palumbi, 1996).

Amplicon site	Primer sequences
Cytochrome oxidase *I* (Approximately 500 bp)	COI f-1 5'-CCTGCCGGAGGAGGTGAYCC-3' (forward) (Reverse) COIe-1 5'-CCAGTAAATAACGGGAATCAGTG-3'(reverse)

After the master mix had been prepared, amplification was performed in a standard thermocycler machine for thirty cycles. Then, the amplified products were visualized by gel electrophoresis of containing 0.8 µl of EtBr. One microlitre (1 µl) of 6X load dye (Promega or Fermentas) was mixed with 2 µl of PCR product and 100 bp DNA ladder plus (GeneRuler^{TM}, Fermentas) as size marker. The electrophoresis was performed for 40 to 45 minutes at 94 V and visualized under UV transluminator and photograph was printed out from the Biorad Documentation System.

Table 2: The PCR master mix for a 25 µl reaction.

Component	per reaction (µl)			
	Volume 1	Volume 2	Volume 3	Volume 4
10 X reaction buffer (25mM)	2.5	2.5	2.5	2.5
$MgCl_2$ (25mM)	1.0	1.5	1.0	1.25
dNTP mix (10mM)	2.5	2.5	2.5	2.5
Primer COIe-1 (10µM)	1.0	1.25	1.5	1.5
Primer COIf-1 (10µM)	1.0	1.25	1.5	1.5
ddH_2O	15.8	14.8	13.8	13.55
DNA template	1.0	1.0	2.0	2.0
Taq Polymerase (5 units per µl)	0.2	0.2	0.2	0.2
Total	25.0			

Table 3: The cycle parameters for PCR.

Steps	Temperature (°C)	Duration (minute)	
Initial denaturation	94	2	
Denaturation	94	1	30 cycles
Annealing	50-54	1	
Extension	72	2	
Final extension	72	5	

3.2.3 DNA Purification and Sequencing

Purification of selected amplified products was done using Fermentas DNA Purification Kit and Genispin PCR Purification Kit following the protocol provided by the manufacturer. The samples were resolved using a 1.5% agarose gel and 50 ml of 1X TAE buffer with EtBr included. Electrophoresis was at 94 V for 45 minutes. A standard 100 bp DNA ladder plus (GeneRuler^{TM}, Fermentas) and 1 µl of purification product mixed with 1 µl of 6X load dye (Promega or Fermentas) were loaded. A set of selected purified products were sent to First Base Company for sequencing. ABI377-96 upgrade gel-based sequencer and ABI3100 capillary sequencer were used.

3.2.4 Sequence Alignment and Phylogenetic Analysis

The program of CHROMAS version 1.45 (McCarthy, 2005) was used to display the fluorescence-based DNA sequence analysis result. Multiple alignments of the nucleotide sequences were done by using the program CLUSTAL X version 1.81 (Thompson *et al.*, 1997) and subsequently aligned manually. The DNA sequences were blasted with DNA sequences of COI from GenBank. The phylogenetic relationship of genus *Tupaia* was reconstructed by using the Maximum Parsimony (MP) and Neighbor-Joining (NJ) methods implemented in

MEGA version 2.1 (Kumar *et al.*, 1994). The NJ clustering was performed using the Kimura two-parameter distance model (Kimura, 1980) while the MP analysis was conducted using close-neighbor interchange option. Phylogenetic confidence was estimated by bootstrapping (Felsenstein, 1985) with 1000 replicate data set. DNA sequences of COI from *Callosciurus notatus* was used as outgroup for this study.

4.0 Results

4.1 Extraction of genomic DNA

The samples of *T. montana* (TK152275, TK152299 and TK152313), *T. tana* (AL1, K4 and TK152285), *T. minor* (K3 and 00829), *T. gracilis* (00171), *T. glis* (K5), *C. notatus* (CN) had obtained positive result. Whereas, *T. minor* (00328) had produced a negative result. From Figure 1 shows that lane 2 had a smearing band while lanes 5 and 7 had a single band; lane 8 had a multiple bands respectively at more than 10 000 bp. However, there were no band observed in lanes 3, 4 and 6.

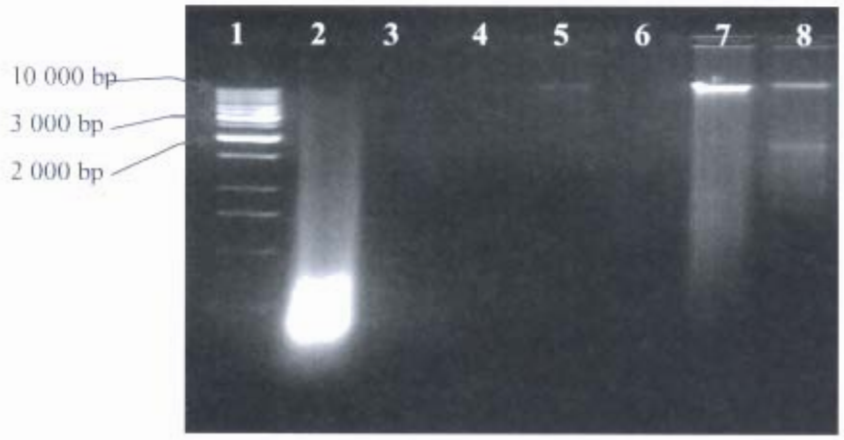

Figure 1: Extraction product of lane 1 = I kb DNA ladder (GeneRuler™, Fermentas), lane 2 = *T. gracilis* (00171), lane 3 = *T. minor* (00328), lane 4 = *T. minor* (00829), and lane 5 = *T. minor* (K3), lane 6 = *T. glis* (K5), lane 7 = *T. glis* (K5) (liver) and lane 8 = *C. notatus* (CN).

From the Figure 2 shows that lane 4 had a single band while samples in lanes 2 and 3 had a smearing background. DNA size was estimated at more than 10 000 bp.

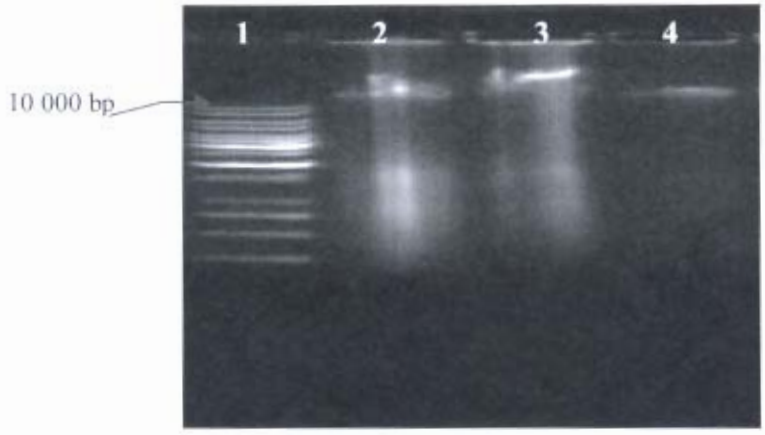

Figure 2: Extraction product of lane 1 = 1 kb DNA ladder (GeneRuler™, Fermentas), lane 2 = *T. tana* (TK152285), lane 3 = *T. tana* (K4), lane 4 = *T. minor* (00829).

Figure 3 shows that lanes 2, 3, 4 and 5 had a single band at the size of more than 10 000 bp.

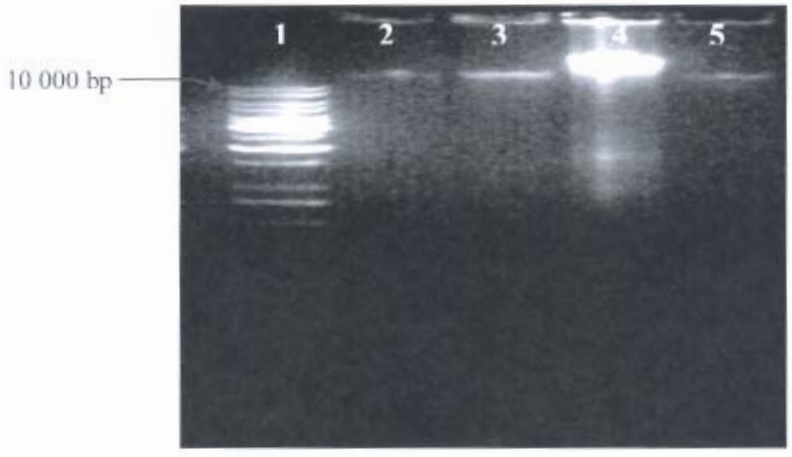

Figure 3: Extraction product of lane 1 = 1 kb DNA ladder (GeneRuler™, Fermentas), lane 2 = *T. tana* (AL1), lane 3 = *T. montana* (TK152275), lane 4 = *T. montana* (TK152299), and lane 5 = *T. montana* (TK152313).